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*J Immunol* 2001; 167:5386-5394; doi: 10.4049/jimmunol.167.9.5386
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Cytokine Requirements for Induction of Systemic and Mucosal CTL After Nasal Immunization

Herman F. Staats,*†‡§¶ Curtis P. Bradney,* William M. Gwinn,† Shawn S. Jackson,‖ Gregory D. Sempowski,*†¶ Hua-Xin Liao,*§¶ Norman L. Letvin,‖ and Barton F. Haynes*§¶

Cholera toxin (CT) is frequently used as an experimental adjuvant intranasally for the induction of systemic and mucosal immunity. However, CT is highly reactogenic and not approved for use in humans. To define the cytokine requirements for the nasal activation of the systemic and mucosal immune system, and to design new adjuvants with efficacy similar to CT, we defined the cytokines that were able to replace CT as a nasal adjuvant for the induction of CTL. BALB/c mice were nasally immunized with an HIV immunogen that contains an MHC class I-restricted CTL epitope and tested for HIV-specific immune responses. We found that combinations of IL-1α plus IL-18, IL-1α plus IL-12, and IL-1α plus IL-12 plus GM-CSF each induced optimal splenocyte anti-HIV CTL responses in immunized mice (range 60–71% peptide-specific 51Cr release). Peak H-2Dk-peptide tetramer-binding T cell responses induced by cytokine combinations were up to 5.5% of CD8+ PBMC. Nasal immunization with HIV immunogen and IL-1α, IL-12, and GM-CSF also induced Ag-specific IFN-γ-secreting cells in the draining cervical lymph node and the lung. The use of IL-1α, IL-12, and GM-CSF as nasal adjuvants was associated with an increased expression of MHC class II and B7.1 on nonlymphocytes within the nasal-associated lymphoid tissue/nasal mucosa. Thus, IL-1α, IL-12, IL-18, and GM-CSF are critical cytokines for the induction of systemic and mucosal CTL after nasal immunization. Moreover, these cytokines may serve as effective adjuvants for nasal vaccine delivery.


Ags delivered via a nasal route first make contact with epithelial cells. IL-1, IL-12, IL-18, GM-CSF, and IFN-γ have all been implicated in a cytokine pathway involved with the induction of MHC class I-restricted CTL via production of IL-1α by the mucosal epithelium (1) and APCs (6), followed by release of IL-18 (7). Both IL-1 and IL-18 are produced by mucosal epithelial cells (1, 8–10). IL-1 induces the production of IL-12 and GM-CSF by resident macrophages and IFN-γ by local NK and T cells (11). IL-18 and IL-12 synergistically induce the production of IFN-γ by local NK cells (7). IFN-γ enhances presentation of MHC class I-restricted Ags (12, 13). T cells responding to specific Ag produce GM-CSF that activates macrophages and dendritic cells to enhance APC activity (14, 15).

We recently reported that IL-1α and IL-1β exhibit mucosal adjuvant activity for the induction of serum IgG and mucosal IgA Ab responses when nasally administered with soluble protein Ags (16). Although IL-1α and IL-1β could support the induction of humoral immunity after nasal immunization with soluble protein Ags, the induction of CTL after nasal immunization was not studied. Because CT is not approved for use in humans, it would be advantageous to replace CT as an adjuvant and to develop an adjuvant combination of cytokines that maximally activated host immunity via nasal immunization. Moreover, in doing so, we would define the cytokine requirements for CTL induction via the nasal route. Therefore, we have evaluated proinflammatory and immunoregulatory cytokines for their ability to induce Ag-specific CTL after nasal immunization with a model soluble protein HIV immunogen.

Materials and Methods

Animals

Female BALB/c mice, 16–18 g, were purchased from Frederick Cancer Research and Developmental Center, National Cancer Institute (Frederick, MD). Animals were housed in filter top cages and provided food and water ad libitum. Procedures for use and care of mice were approved by Duke University’s Institutional Animal Care and Use Committee.
Immunization
Mice were intranasally (i.n.) immunized, as previously described (16–18). Briefly, mice (three to five mice per group) were immunized i.n. with the indicated amount of HIV immunogen and the indicated adjuvant in a total volume of 15 μl sterile distilled water (7.5 μl/mouse), while mice were under isoflurane anesthesia (IsoFlo, USP, SOLVAY Animal Health, Mendota Heights, MN). The mucosal adjuvant CT was obtained from List Biological Laboratories (Campbell, CA). Recombinant murine cytokines were purchased from PeproTech (Rocky Hill, NJ), BioSource International (Camarillo, CA), or Chemicon (Temecula, CA).

HIV synthetic peptides
Synthetic HIV peptides C4-V3HIB and C4-V3MN were used as HIV immunogens (17, 19–21). The amino acid sequence for C4-V3HIB is KQINMWQEVKAMYATRPNYNKRKRIHPGPRGAFVTI. The amino acid sequence for C4-V3MN is KQINNWQEVKAMYATRPNYNKRKRIHPGPRGAFVTI (21). Smaller synthetic peptides from the gp120 V3 loop of HIV-1HIB (RPGPRGAFVTI) and HIV-1MN (RIGHPGPRGAFVTTK) containing the H-2Dd-restricted CTL epitopes were used for in vitro restimulation of CTL effector cells, labeling of CTL target cells, and antigenic stimulation of splenocytes in the IFN-γ ELISPOT assay. Peptides used for immunization of mice were purchased from SynPep (Dublin, CA) and purchased as single species by HPLC and verified by mass spectroscopy. Peptides used for in vitro assays were purchased from SYNPEH as HPLC-purified peptides with >90% purity. Peptide mass was verified by mass spectroscopy.

CTL assay
Restimulation of effector cells. BALB/c splenocytes were removed, purified by lymphocyte separation medium (ICN Biomedicals, Aurora, OH), and used as effector cells to monitor HIV-specific CTL responses. Splenocyte numbers were determined in a CTL media (RPMI 1640, 10% FBS, HEPES, penicillin/streptomycin, nonessential amino acids, essential amino acids, 2-ME, 2 nM NaOH, sodium pyruvate) at a cell density of 1 × 10^6 cells/ml. Effector cells were added to wells in a 24-well plate (750 μl/well), followed by the addition of 1 ml CTL media containing the appropriate CTL epitope peptide at 1.75 μg/ml to each well to give a final peptide concentration of 1 μg/ml. Splenocytes from naive mice were also restimulated to determine whether in vitro restimulation induced peptide-specific CTL. Cells were incubated in 10% CO₂ for 7 days. Day 2 or 3, 500 μl CTL media containing murine rIL-2 (24 U/ml) were added to each well of CTL effector cells to give a final concentration of 10 U/ml murine rIL-2. Effector cells were tested in a chromium release assay after restimulation for 7 days.

Chromium release assay. A standard chromium release assay was used to monitor CTL activity. Briefly, target cells were pulsed with 100 μCi/ml ^51Cr or 40 μg/ml CTL epitope peptide for 3–4 h before use in the 4-h chromium release assay. Percent specific lysis was calculated as follows: ((experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm)) × 100. Results are presented as peptide-specific lysis, calculated by subtracting the percent specific lysis of control target cells from the percent specific lysis of the peptide-pulsed target cells at the same E:T ratio.

IFN-γ ELISPOT
Millipore MAHA S45 96-well multiscreen plates were coated with 50 μl/well anti-mouse IFN-γ capture Ab (catalog 18180D; BD PharMingen, San Diego, CA) diluted to 5 μg/ml in sterile PBS. Plates were incubated overnight at 4°C or 3 h at 37°C. After incubation, plates were washed three times with 100 μl/well PBS/2% FCS, 100 μl/well, and then incubated with CTL epitope peptide at 100 μg/ml for at least 30 min. Blocking media were removed, and 100 μl cells (CTL epitope peptide at 1 μg/ml final concentration) were added to appropriate wells. Cells were added at concentrations to give 5 × 10^3, 2.5 × 10^3, and 1.25 × 10^3 cells/well. Plates were incubated at 37°C, 10% CO₂ in air, humidified atmosphere for 36–48 h after incubation. Plates were washed three times with 100 μl PBS, followed by three times with 100 μl PBS. Anti-IFN-γ detection Ab (BD PharMingen, 18112D) was diluted to 5 μg/ml in PBS-10% FBS, and 50 μl well was added to each well and incubated at room temperature for 3 h. After incubation, plates were washed six times with PBS-Tween, and streptavidin-HRP (BD PharMingen; 3047E) diluted 1/500 in PBS-10% FBS was added to each well (50 μl/well) and incubated at room temperature for 1 h. After incubation, plates were washed six times with PBS-Tween and developed with 100 μl/well 3-aminophenylcarbazole substrate (according to manufacturer’s instructions; Pierce Chemical, Rockford, IL) at room temperature for up to 1 h. The reaction was stopped by rinsing plate in tap water.

The plastic support from back of 96-well filter plates was removed and plates were air dried overnight before spots were counted with a Leica (Deerfield, IL) StereoZoom 5 microscope with fiber optic ring lamp. The number of peptide-specific IFN-γ producing cells per 10^6 cells was calculated by subtracting the number of IFN-γ spot-forming cells (SFC)/10^6 detected in the absence of CTL peptide stimulation from the number of IFN-γ SFC/10^6 detected in the presence of CTL peptide stimulation.

HMC class I-peptide tetramer-binding assay; tetramer H-2Dd/p18 complex formation
A pET-3d vector containing DNA coding for the soluble domain of H-2Dd with a 3’ BirA substrate peptide, generated as described by Altman et al. (22) and Kuroda et al. (23), was kindly provided by C. Bergmann (University of Southern California, Los Angeles, CA) for tetramer production. Tetramer conditions were expressed in cultures of XA90 (University of Southern California, Los Angeles, CA) for tetramer production and Kuroda et al. (22) and Kuroda et al. (23), was kindly provided by C. Bergmann (University of Southern California, Los Angeles, CA) for tetramer production.

Isolation of lung cells
Cells within the circulatory system of the lung were removed by injecting digestion media (RPMI 1640, 5% FBS, HEPES, penicillin/streptomycin, 2.5 mg/ml collagenase A; Roche Molecular Biochemical 1088 785, Indianapolis, IN) and 34 U/ml DNase I, grade II (Roche Molecular Biochemical 104 159) into the right ventricle of the heart until the lung became white. The lung was then removed and placed in digestion media and minced into small pieces (<2 mm³) using scissors. Lung tissue was then placed in a 50-ml Erlenmeyer flask with 25 ml digestion media and a stir bar and placed on a magnetic stirrer housed in an incubator at 37°C and 5% CO₂. The lung tissue was digested for up to 2 h and then filtered through a 40-μm cell strainer (catalog 35-2340; Falcon, Franklin Lakes, NJ). Isolated lung cells were pelleted, resuspended in 10 ml room temperature 44% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ), and then layered over 10 ml lymphocyte separation media (ICN Biomedicals) in a 50-ml centrifuge tube and centrifuged for 20 min at 1800 rpm. Cells at the Percoll-lymphocyte separation media interface were collected, washed, and used in the IFN-γ ELISPOT assay.

Staining and phenotypic analysis of p18-specific CD8+ T cells
A total of 0.1–0.2 μg PE-labeled tetrameric complex was used in conjunction with anti-CD8α (clone 53-6.7; BD PharMingen) conjugated to PerCP to stain 100 μl blood processed, as indicated below. Peripheral blood samples were diluted in RPMI (4 U/ml sodium heparin). Samples were washed with RPMI and lysed in 144 mM NH₄ Cl, 17 mM Tris buffer (pH 7.2). The samples were resuspended (to 100 μl/sample) for staining in PBS/2% FCS. Samples were incubated with the tetrameric complexes, followed by incubation with anti-CD8α (15 min at room temperature). Cells were washed with PBS/2% FCS, resuspended in 0.5 ml PBS containing 1% paraformaldehyde, and analyzed on a BD Biosciences (Mountain View, CA) FACSCalibur.

Isolation of lung cells
Cells within the circulatory system of the lung were removed by injecting digestion media (RPMI 1640, 5% FBS, HEPES, penicillin/streptomycin, 2.5 mg/ml collagenase A; Roche Molecular Biochemical 1088 785, Indianapolis, IN) and 34 U/ml DNase I, grade II (Roche Molecular Biochemical 104 159) into the right ventricle of the heart until the lung became white. The lung was then removed and placed in digestion media and minced into small pieces (<2 mm³) using scissors. Lung tissue was then placed in a 50-ml Erlenmeyer flask with 25 ml digestion media and a stir bar and placed on a magnetic stirrer housed in an incubator at 37°C and 5% CO₂. The lung tissue was digested for up to 2 h and then filtered through a 40-μm cell strainer (catalog 35-2340; Falcon, Franklin Lakes, NJ). Isolated lung cells were pelleted, resuspended in 10 ml room temperature 44% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ), and then layered over 10 ml lymphocyte separation media (ICN Biomedicals) in a 50-ml centrifuge tube and centrifuged for 20 min at 1800 rpm. Cells at the Percoll-lymphocyte separation media interface were collected, washed, and used in the IFN-γ ELISPOT assay.
Phenotypic characterization of nasal-associated lymphoid tissue (NALT) and nasal mucosa cells using flow cytometry

Cells were harvested from the NALT and nasal mucosa as follows. The skin was removed from the head, and the lower jaw was removed. Scissors were used to cut the skull behind the eyes, removing the posterior portion of the snout from the rest of the skull. The bony skull was then cut from the opening of each nostril to the eye socket on each side of the skull. The nasal septum was cut, and the top portion of the skull was removed, leaving the palate and associated nasal tissues. Scissors were used to make a section cut to remove the upper incisors. Cells in the NALT and nasal mucosa area were removed by mechanically scraping the area immediately above the soft palate that contains the NALT structures (24). Cells from mucosa area were removed by mechanically scraping the area immediately above the soft palate that contains the NALT structures (24). Cells from five mice per group were pooled in media (RPMI 1640, 5% FBS). Cells were filtered using a 40-μm cell strainer (Falcon; catalog 35-2340). RBC were removed using ammonium chloride lysis buffer. NALT/nasal mucosa cells were then washed, incubated on ice with unlabeled mouse IgG2a (10 μg/10⁶; Southern Biotechnology Associates, Birmingham, AL) cells to block Fc receptors, washed, and then resuspended to 3 × 10⁸ cells/ml. Fluorescently labeled and biotinylated mAbs (BD PharMingen) were diluted in wash buffer (PBS, 1% FBS, 0.1% Kathon), and a total volume of 50 μl containing 0.25 μg of each appropriate Ab was added per well of a 96-well round-bottom plate. A total of 150,000 cells in 50 μl media was added per well (to give a 100 μl final volume), gently mixed, and stained for 30 min at 4°C in the dark. Cells were stained with FITC anti-CD3, PE anti-pan NK, CyChrome anti-CD8, FITC anti-B220, FITC anti-CD11c, or APC anti-Gr-1. Appropriate negative (isotype) and positive controls for each fluorescent label were also included. For analysis of MHC class II, B7.1, and B7.2, cells were triple stained with FITC anti-CD3; PE anti-CD19; and either biotinylated anti-MHC class II, anti-B7.1, or anti-B7.2 for 30 min in the dark at 4°C. After washing, secondary labeling with streptavidin-APC was performed for 20 min in the dark at 4°C. Cells were washed after staining with wash buffer and resuspended in 200 μl/well fluorescent fixative (PBS, 1% formaldehyde, 0.1% Kathon). Cells were transferred to polystyrene tubes containing an additional 300 μl fluorescent fixative (0.5 ml final volume), gently mixed, and analyzed with a FACSVantage SE from BD Biosciences using CellQuest software. To distinguish between lymphocytes and nonlymphocytes, analysis of MHC class II, B7.1, and B7.2 was performed using CD3⁺ or CD19⁺ or CD3⁻ and CD19⁻ gated cells. All viable cells, based on forward and side scatter, were gated to include all cell populations present in the NALT/nasal mucosa.

Statistical analysis

Statistical analysis was determined using t test analysis. Because the comparison of activity between control groups (nasal immunization with peptide without adjuvant to nasal immunization with peptide plus adjuvant) was planned a priori, t tests were performed to compare each experimental group with the control group (25, 26). The level of significance used was 0.05. Error bars represent SD.

Results

Cytokines are able to support the induction of spleen HIV-1-specific CTL and IFN-γ secretion after nasal administration with an HIV immunogen

BALB/c mice were nasally immunized with the C4-V3MN HIV-1 immunogen at 1, 10, or 100 μg ± cytokines on days 0, 7, 14, and 28 to identify cytokines involved with the induction of HIV-1-specific CTL. Cytokines tested included IL-1α, IL-12, IL-18, IL-12, and IL-18, and GM-CSF (Table I). Nasal administration of adjuvants alone or C4-V3MN alone induced only background levels of ³¹¹Cr release (Fig. 1). When 10 μg C4-V3MN was used as the nasal immunogen, CT was able to induce HIV-specific cell lysis that was significantly elevated compared with mice nasally immunized with 10 μg C4-V3MN alone (61.4 ± 2.66% vs 19.41 ± 2.65%.

<table>
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<tr>
<th>Route of Immunization</th>
<th>Adjuvant (Dose)</th>
<th>Immunogen (Dose)</th>
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<tr>
<td>s.c.</td>
<td>CFA/IFA</td>
<td>C4-V3MN (10 μg)</td>
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<tr>
<td>Nasal</td>
<td>None</td>
<td>C4-V3MN (10 μg)</td>
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<tr>
<td>Nasal</td>
<td>CT (1 μg)</td>
<td>C4-V3MN (10 μg)</td>
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<td>C4-V3MN (10 μg)</td>
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6.95% HIV peptide-specific lysis, respectively, \( p = 0.0003 \) (Fig. 1). Nasal immunization with 10 \( \mu \text{g} \) C4-V3\(_{\text{IMM}} \) and CT was also the group with the absolute highest level of HIV-specific cell lysis (Fig. 1). Cytokines that were able to induce significant CTL responses \( (p < 0.02) \) include IL-1\( \alpha \), IL-18, and IL-12 and IL-18 when nasally administered with 100 \( \mu \text{g} \) HIV immunogen. Nasal immunization with 100 \( \mu \text{g} \) C4-V3\(_{\text{IMM}} \) alone induced 13.67 \( \pm \) 1.7% peptide-specific lysis, while that addition of IL-1\( \alpha \), IL-18, or IL-12 and IL-18 increased peptide-specific lysis to 42.44 \( \pm \) 14.2, 23.96 \( \pm \) 5.5, and 46.29 \( \pm \) 6.6% peptide-specific lysis, respectively. GM-CSF nor IL-12 alone induced significant increases in CTL lytic activity. Therefore, IL-1\( \alpha \), IL-18, and the combination of IL-12 and IL-18 were important for the induction of peptide-specific CTL after nasal immunization.

To quantify the magnitude of the induced Ag-specific T cell population after nasal immunization, splenocytes from mice immunized with C4-V3\(_{\text{IMM}} \) \( \pm \) adjuvants were evaluated for Ag-specific IFN-\( \gamma \) secretion using the ELISPOT assay (Fig. 2). As observed with the CTL lysis, the group with the absolute highest frequency of IFN-\( \gamma \)-secreting cells was immunized with 10 \( \mu \text{g} \) C4-V3\(_{\text{IMM}} \) and CT (Fig. 2). When 100 \( \mu \text{g} \) C4-V3\(_{\text{IMM}} \) was used as the immunogen, CT, IL-1\( \alpha \), IL-18, IL-12 and IL-18, and GM-CSF induced IFN-\( \gamma \)-secreting cell frequencies \((199.7 \pm 70.2, 150.5 \pm 89.5, 49.5 \pm 23.4, 89 \pm 51.2, \) and \( 137.7 \pm 73.1 \) IFN-\( \gamma \) SFC/\( 10^6 \) splenocytes, respectively) significantly \( (p < 0.03) \) above those detected in mice immunized with 100 \( \mu \text{g} \) C4-V3\(_{\text{IMM}} \) only \((13.67 \pm 4.5 \) IFN-\( \gamma \) SFC/\( 10^6 \) splenocytes). Thus, IL-1\( \alpha \) alone, IL-18 alone, IL-12 and IL-18 in combination, and GM-CSF alone were important for nasal induction of Ag-specific IFN-\( \gamma \)-secreting cells.

**More complex combinations of cytokines support induction of peptide-specific CTL, IFN-\( \gamma \)-producing T cells, and H-2D\( ^k \) peptide tetramer-positive CD8\( ^+ \) T cells comparable with CT**

Next, more complex combinations of cytokines were tested for their ability to induce HIV-specific CTL, IFN-\( \gamma \) secretion, and tetramer binding comparable with those induced with the use of CT. Because the highest level of Ag-specific CTL activity and IFN-\( \gamma \) secretion was detected after i.n. immunization with 10 \( \mu \text{g} \) C4-V3\(_{\text{IMM}} \) and CT (Figs. 1 and 2), the 10 \( \mu \text{g} \) dose of C4-V3\(_{\text{IMM}} \) was used for the remaining studies. Mice were immunized nasally with 10 \( \mu \text{g} \) C4-V3\(_{\text{IMM}} \) alone or in the presence of CT or cytokine combinations. As previously observed, nasal immunization with 10 \( \mu \text{g} \) C4-V3\(_{\text{IMM}} \) and CT induced CTL lytic activity \((40.96 \pm 0.48\% \) peptide-specific lysis) that was significantly increased compared with the CTL activity detected in naive animals or animals immunized with C4-V3\(_{\text{IMM}} \) alone \((14.62 \pm 2.72\% \) and 15.18 \( \pm \) 0.37\% peptide-specific lysis, respectively, \( p < 0.00001 \)) (Fig. 3). Cytokine combinations that induced CTL lytic activities that were significantly increased compared with immunization with C4-V3\(_{\text{IMM}} \) alone included IL-1\( \alpha \) plus IL-18 \( (p < 0.00001) \), GM-CSF plus IL-12 plus IL-18 \( (p < 0.05) \), IL-1\( \alpha \) plus GM-CSF \( (p < 0.001) \), IL-1\( \alpha \) plus IL-12 \( (p < 0.00001) \), IL-1\( \alpha \) plus IL-18 \( (p < 0.00001) \), and IL-1\( \alpha \) plus GM-CSF \( (p < 0.001) \).
plus IL-12 plus IL-18 plus GM-CSF ($p < 0.0002$), and IL-1α plus IL-12 plus GM-CSF ($p < 0.0006$) (Fig. 3). Importantly, the combinations of IL-1α plus GM-CSF, IL-1α plus IL-12, IL-1α plus IL-18, IL-1α plus IL-12 plus GM-CSF, and IL-1α plus IL-12 plus IL-18 plus GM-CSF induced the highest CTL lytic activities (59.55 ± 10.5, 59.6 ± 3.7, 64.33 ± 5.2, 71.66 ± 11.7, 67.01 ± 7.1% peptide-specific lysis, respectively) that were all significantly increased compared with that induced by nasal immunization with C4-V3 immun and CT ($p < 0.02$) (Fig. 3).

The frequency of Ag-specific IFN-γ-secreting cells in splenocytes after nasal immunization with immunogen and complex cytokine combinations was next determined using the ELISPOT technique. Nasal immunization with C4-V3 immun and CT induced $152.6 \pm 20.9$ IFN-γ-secreting cells/10⁶ splenocytes, while nasal immunization with C4-V3 immun only induced $3.66 \pm 6.35$ IFN-γ-secreting cells/10⁶ splenocytes. Significant increases in peptide-specific IFN-γ secretion were observed in all groups, except GM-CSF and IL-12 used alone ($p < 0.05$) (Fig. 4). The frequency of peptide-specific IFN-γ-secreting splenocytes was greater than 200/10⁶ splenocytes when IL-1α plus IL-18, IL-1α plus IL-12 plus GM-CSF, IL-1α plus IL-18 plus GM-CSF, and IL-1α plus IL-12 plus IL-18 plus GM-CSF were used as adjuvants ($236 \pm 117, 209 \pm 46, 227 \pm 107,$ and $231 \pm 68$ IFN-γ SFC/10⁶ splenocytes, respectively) (Fig. 4).

H-2Dᵇ-HIV peptide tetramers were used in flow cytometric assays to identify the percentage of CD8⁺ cells in the peripheral blood that were specific for the HIV envelope V3 P18 HIV CTL epitope that was in our model HIV immunogen. The percentage of tetramer-positive cells in the CD8⁺ population of the peripheral blood of animals immunized with peptide alone was not increased over the percentage of tetramer-positive cells detected in naive mice (0.3 ± 0.14% and 0.45 ± 0.31%, respectively; $n = 3$) (Fig. 5). The use of CT as an adjuvant significantly increased the percentage of tetramer⁺ cells to $2.9 \pm 0.7\%$ of the CD8⁺ PBMC ($p < 0.002$ compared with tetramer⁺ cells in mice immunized with peptide alone). Cytokines and cytokine combinations that significantly increased the percentage of tetramer⁺ cells in the CD8⁺ PBMC pool include IL-1α ($p < 0.0004$), GM-CSF ($p < 0.02$), IL-12 plus IL-18 ($p < 0.02$), GM-CSF plus IL-12 plus IL-18 ($p < 0.004$), IL-1α plus GM-CSF ($p < 0.004$), IL-1α plus IL-12 ($p < 0.02$), IL-1α plus IL-18 ($p < 0.0002$), IL-1α plus IL-12 plus GM-CSF ($p < 0.02$), IL-1α plus IL-18 plus GM-CSF ($p < 0.0006$), and IL-1α plus IL-12 plus IL-18 plus GM-CSF ($p < 0.0004$) (Fig. 5). The combination of IL-1α plus IL-18 induced the absolute highest percentage of tetramer⁺ cells in the CD8⁺ PBMC pool after nasal immunization with peptide immunogen in the presence of cytokine(s). BALB/c mice (three to four mice per group) were immunized nasally on days 0, 7, 14, and 28 with 10 μg C4-V3 immun peptide alone or in combination with CT or select cytokines (Table I). Mice were boosted on day 42 with 10 μg peptide only. Mice were lightly anesthetized with isoflurane before 7.5 μl vaccine formulation was instilled into each nostril. Splenocytes were isolated at day 51 and used in an IFN-γ ELISPOT assay. * Statistically increased frequency of IFN-γ-producing cells compared with animals nasally immunized with peptide alone ($p \leq 0.05$). No groups were significantly increased compared with animals immunized with CT and peptide.

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Induction of peptide-specific CTL after nasal immunization with peptide immunogen in the presence of cytokine(s). BALB/c mice (three to four mice per group) were immunized nasally on days 0, 7, 14, and 28 with 10 μg C4-V3 immun peptide alone or in combination with CT or select cytokines (Table I). Mice were boosted on day 42 with 10 μg peptide only. Mice were lightly anesthetized with isoflurane before 7.5 μl vaccine formulation was instilled into each nostril. Splenocytes were isolated at day 51 and restimulated in vitro for 7 days before being used in a chromium release CTL assay. *, Statistically increased CTL activity compared with animals nasally immunized with peptide alone ($p \leq 0.05$).

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Induction of peptide-specific IFN-γ SFC after nasal immunization with peptide immunogen in the presence of cytokine(s). BALB/c mice (three to four mice per group) were immunized nasally on days 0, 7, 14, and 28 with 10 μg C4-V3 immun peptide alone or in combination with CT or select cytokines (Table I). Mice were boosted on day 42 with 10 μg peptide only. Mice were lightly anesthetized with isoflurane before 7.5 μl vaccine formulation was instilled into each nostril. Splenocytes were isolated at day 51 and used in an IFN-γ ELISPOT assay. *, Statistically increased frequency of IFN-γ-producing cells compared with animals nasally immunized with peptide alone ($p \leq 0.05$). No groups were significantly increased compared with animals immunized with CT and peptide.

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Induction of MHC class I peptide tetramer-binding CD8⁺ PBMC after nasal immunization with peptide immunogen in the presence of cytokine(s). BALB/c mice (three to four mice per group) were immunized nasally on days 0, 7, 14, and 28 with 10 μg C4-V3 immun peptide alone or in combination with CT or select cytokines (Table I). Mice were boosted on day 42 with 10 μg peptide only. Mice were lightly anesthetized with isoflurane before 7.5 μl vaccine formulation was instilled into each nostril. PBMC were isolated at day 51 and stained for the presence of CD8 and H-2Dᵇ-g C4-V3 peptide alone or in combination with CT or select cytokines (Table I). Mice were boosted on day 42 with 10 μg peptide only. Mice were lightly anesthetized with isoflurane before 7.5 μl vaccine formulation was instilled into each nostril. Splenocytes were isolated at day 51 and used in an IFN-γ ELISPOT assay. *, Statistically increased frequency of IFN-γ-producing cells compared with animals nasally immunized with peptide alone ($p \leq 0.05$). No groups were significantly increased compared with animals immunized with CT and peptide.
with 5.52 ± 0.71%. The percentage of tetramer+ cells induced by IL-1α plus IL-18 was significantly increased compared with the percentage of tetramer+ cells induced by CT (p < 0.006).

To determine the reproducibility of the adjuvant effect of nasal cytokine combinations, BALB/c mice were immunized nasally with the C4-V3MN immunogen, a model HIV immunogen designed from a second HIV strain, HIV-1 MN. Nasal immunization with peptide alone did not induce CTL lytic activity that was greater than that observed in naive mice (Fig. 6). When nasally administered with the C4-V3MN immunogen, CT and all other cytokines or cytokine combinations tested induced Ag-specific CTL lytic responses that were significantly increased compared with responses induced by nasal immunization with C4-V3MN alone (p < 0.03). The highest level of Ag-specific CTL activity induced by nasal immunization with C4-V3MN was in the presence of IL-α plus IL-12 plus IL-18 plus GM-CSF with 41.47 ± 9.53% Ag-specific lysis when tested at a 20:1 E:T ratio (Fig. 6). IL-1α plus IL-12 plus GM-CSF and CT induced 35.12 ± 7.9% and 32.07 ± 3.44% specific release, respectively, at 20:1 E:T ratio.

**Nasal immunization with HIV immunogen and cytokine adjuvants induces HIV-specific IFN-γ-secreting cells in systemic and mucosal tissues**

The induction of HIV-specific cell-mediated immunity at mucosal surfaces may be important for vaccine-induced protection against mucosal transmission of HIV. We therefore determined whether nasal immunization with HIV immunogen and the cytokine adjuvant combination of IL-1α, IL-12, and GM-CSF induced HIV-specific IFN-γ-secreting cells in the draining cervical lymph node (the lymph node that drains the NALT), spleen, and lung (as a representative mucosal tissue). Nasal immunization with 10 μg C4-V3MN and IL-1α, IL-12, and GM-CSF induced 311 ± 56.7, 356 ± 50.9, and 318 ± 145.2 HIV-specific IFN-γ SFC/106 cells in the cervical lymph node, spleen, and lung, respectively (p ≤ 0.01 vs peptide only; Fig. 7). Nasal immunization with peptide alone induced IFN-γ SFC responses less than three IFN-γ SFC/106 cells, while naive mice had no detectable HIV-specific IFN-γ SFC responses in any tissue (Fig. 7).

**FIGURE 6.** Induction of peptide-specific CTL after nasal immunization with peptide immunogen in the presence of cytokine(s). BALB/c mice (three to four mice per group) were immunized nasally on days 0, 7, 14, and 28 with 10 μg C4-V3MN peptide alone or in combination with CT or select cytokines (Table I). Mice were boosted on day 42 with 10 μg peptide only. Mice were lightly anesthetized with isoflurane before 7.5 μl vaccine formulation was instilled into each nostril. Splenocytes were isolated at day 51 and restimulated in vitro for 7 days before being used in a chromium release CTL assay. *Statistically increased CTL activity compared with animals nasally immunized with peptide alone (p ≤ 0.05).

**FIGURE 7.** Induction of peptide-specific IFN-γ SFC in systemic and mucosal tissues after nasal immunization with peptide immunogen in the presence of IL-1α, IL-12, and GM-CSF. BALB/c mice (15 mice per group) were immunized nasally on days 0, 7, 14, and 28 with 10 μg C4-V3MN peptide alone or in combination with IL-1α, IL-12, and GM-CSF (Table I). Mice were lightly anesthetized with isoflurane before 7.5 μl vaccine formulation was instilled into each nostril. Splenocytes, cervical lymph nodes, and lung cells were isolated from five mice/group on days 34, 35, and 36, pooled, and used in an IFN-γ ELISPOT assay. *Statistically increased frequency of IFN-γ-producing cells compared with animals nasally immunized with peptide alone or naive animals (p ≤ 0.05).

**Nasal immunization with HIV immunogen and cytokine adjuvants is associated with an increase in CD3+ cells and increased expression of MHC class II and B7.1 in NALT/nasal mucosal cells**

To determine whether there was an association between 1) the use of cytokines as mucosal adjuvants, 2) the induction of HIV-specific IFN-γ SFC responses, and 3) changes in the cellular populations within the NALT/nasal mucosal, cells were isolated from the NALT/nasal mucosa of mice used in the experiment for Fig. 7 and analyzed via flow cytometry for the expression of CD3, B220, DX5 (pan-NK), CD11c, Gr-1, MHC class II, B7.1, and B7.2 (Table II). Mice immunized with HIV immunogen plus IL-1α, IL-12, and GM-CSF had 34.25 ± 2.77% CD3+ cells in the cervical lymph node, spleen, and lung, respectively (p ≤ 0.05). The use of IL-1α, IL-12, and GM-CSF was also associated with a significant decrease in B220+ cells since mice immunized with immunogen only had 29.5 ± 2.79% B220+ cells, while mice immunized with immunogen had 21.4 ± 3.62% CD3+ cells (p ≤ 0.05). The use of IL-1α, IL-12, and GM-CSF had 34.25 ± 2.77% CD3+ cells in the NALT/nasal mucosal, while mice immunized with HIV immunogen had 21.4 ± 3.62% CD3+ cells (p ≤ 0.05). The lymphocyte (CD3+ or CD19+) and nonlymphocyte (CD3− and CD19−) populations were also analyzed for the expression of MHC class II, B7.1, and B7.2 to determine whether up-regulation of molecules associated with Ag presentation and costimulation was associated with the use of IL-1α, IL-12, and GM-CSF as mucosal adjuvants. The use of IL-1α, IL-12, and GM-CSF was associated with an increase in both the percentage of positive cells and the mean fluorescence intensity (MFI) of cells in the nonlymphocyte gate when assayed for reactivity with Abs to MHC class II, B7.1, and B7.2, although only the changes in MHC class II and B7.1 were statistically significant (Table II). No changes in MHC class II, B7.1, or B7.2 expression were detected in the lymphocyte population (data not shown). The percentage of nonlymphocytes...
expressing MHC class II increased from 12.97 ± 3.26% to 29.52 ± 3.44% when IL-1α, IL-12, and GM-CSF were used as a mucosal adjuvant (p ≤ 0.05). The MFI for MHC class II staining on nonlymphocytes increased from 74.02 ± 30.97 to 205.36 ± 39.85 with the use of IL-1α, IL-12, and GM-CSF (p ≤ 0.05). The number of nonlymphocytes expressing B7.1<sup>b</sup> also increased from 7.89 ± 3.90% to 13.22 ± 1.87% with the use of IL-1α, IL-12, and GM-CSF (p = 0.05). The MFI for B7.1 expression on nonlymphocytes increased from 6.17 ± 5.30 to 13.6 ± 2.93 when IL-1α, IL-12, and GM-CSF were used as a mucosal adjuvant (p = 0.05).

### Discussion

In this study, we report that combinations of cytokines such as IL-1α plus IL-12 plus GM-CSF and IL-1α plus IL-18 were able to substitute for CT as a nasal adjuvant when administered with a model HIV immunogen and support the induction of Ag-specific CTL, IFN-γ-producing cells, and H-2D<sup>d</sup>-tetramer-binding, CD8<sup>+</sup> peripheral blood T cells. Importantly, nasal immunization with immunogen and combinations of IL-1α plus GM-CSF, IL-1α plus IL-12, IL-1α plus IL-18, IL-1α plus IL-12 plus GM-CSF, or IL-1α plus IL-12 plus IL-18 plus GM-CSF induced CTL responses that were significantly greater than those induced by nasal immunization with peptide and CT.

Nasal immunization with Ag alone may induce Ag-specific mucosal tolerance (27–29), while nasal immunization with Ag and adjuvant has the potential to induce Ag-specific responses, including systemic and mucosal IgG and IgA and systemic CT (16, 17, 30). Adjuvants induce cytokine production and release at the site of immunization that play a crucial role in the induction of Ag-specific immune responses. Mucosal epithelial cells are able to produce numerous cytokines that may be involved with the induction and regulation of immunity, including IL-1, IL-6, IL-18, and IL-8 (31–35). Mucosal epithelial cells are also responsive to local cytokine expression since they express receptors IL-1, IL-6, and GM-CSF (36, 37).

To our knowledge, this is the first report of the ability of cytokines alone to induce CTL after mucosal immunization with Ag. The cytokines GM-CSF and IL-12 have been reported to augment CT induction after intraepithelial immunization of mice with an HIV peptide immunogen when CT was used as an adjuvant (38). We previously determined that IL-1α and IL-1β were able to support the induction of Ag-specific serum IgG and mucosal IgA as well as CT after nasal immunization of mice (16). Others have reported that lymphotactin was able to support the induction of Ag-specific humoral and Th responses after nasal administration with the protein Ag OVA (39). IL-12 has also been reported to have adjuvant activity for the induction of humoral and Th responses when nasally administered with Ag (40, 41). However, in these studies, IL-12 was formulated with liposomes and administered on days 0, 3, 7, 10, 14, and 17, while Ag was administered on days 0, 7, and 14. In our present study, cytokines were formulated with Ag in distilled water and coadministered at the same time.

Some cytokines evaluated in our study were active when delivered i.n., while others were not. For example, IL-1, IL-18, and GM-CSF exhibit activity for the induction of CTL when delivered i.n. with 100 μg C4-V3<sub>IIIb</sub> HIV immunogen, while IL-12 was not. This may be explained by the finding that mucosal epithelial cells express receptors for IL-1 and GM-CSF (37, 42). Therefore, IL-1 and GM-CSF are able to bind to their specific receptors on the mucosal epithelial cells, exert their biological activity, and support the induction of CTL. A possible explanation of the lack of adjuvant activity observed with the use of IL-12 alone is that the mucosal epithelial cells may not express receptors for IL-12 (43). We found IL-12 enhanced the induction of CTL when administered with IL-1α. One possible explanation to this latter observation is that IL-1 increases the permeability of nasal mucosa and allows IL-12 to cross the mucosal epithelium and exert its biological activity in the NALT. Although others have reported that IL-12 exhibits mucosal adjuvant activity after nasal administration (as mentioned previously) (41), the use of liposomes in the IL-12 formulation along with a higher dose of IL-12 (1 μg) and more frequent administration may have permitted the IL-12 to be active when administered by the nasal route.

Others have reported that cytokines were able to regulate the immune responses induced when systemically administered with HIV immunogens similar to the immunogens used in this study (44). In this previously published study, cytokines were formulated with peptide in incomplete Seppic adjuvant (mineral oil plus mannoside) and administered s.c. Subcutaneous administration of immunogen and cytokines determined that GM-CSF was the most effective single cytokine for enhancement of cellular and humoral immunity (44). Interestingly, this study also observed that IL-1β significantly suppressed the CTL response (44). The difference between this published study and our current study is likely

### Table II. Phenotype of NALT/nasal mucosa cells after nasal immunization with C4-V3<sub>IIIb</sub> + cytokine adjuvants<sup>a</sup>

<table>
<thead>
<tr>
<th>Cell Surface Marker</th>
<th>Nasal Immunization with Peptide Only</th>
<th>Nasal Immunization with Peptide + IL-1α, IL-12, and GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent positive cells</td>
<td>MFI</td>
</tr>
<tr>
<td>CD3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.4 ± 3.62</td>
<td>NT</td>
</tr>
<tr>
<td>B220</td>
<td>29.5 ± 2.79</td>
<td>NT</td>
</tr>
<tr>
<td>DX5</td>
<td>2.19 ± 0.86</td>
<td>NT</td>
</tr>
<tr>
<td>CD11c&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.47 ± 1.45</td>
<td>NT</td>
</tr>
<tr>
<td>Gr-1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.48 ± 0.86</td>
<td>NT</td>
</tr>
<tr>
<td>MHC II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-lymphocytes</td>
<td>12.97 ± 3.26</td>
<td>74.02 ± 30.97</td>
</tr>
<tr>
<td>B7.1</td>
<td>7.89 ± 3.90</td>
<td>6.17 ± 5.30</td>
</tr>
<tr>
<td>Non-lymphocytes</td>
<td>4.70 ± 2.35</td>
<td>2.22 ± 1.92</td>
</tr>
</tbody>
</table>

<sup>a</sup> Increased expression of costimulatory molecules on NALT/nasal mucosa cells after nasal administration of HIV immunogen plus IL-1α, IL-12, and GM-CSF. BALB/c mice (15 mice per group) were immunized nasally on days 0, 7, 14, and 28 with 10 μg C4-V3<sub>IIIb</sub> peptide alone or in combination with IL-1α, IL-12, and GM-CSF (Table I). Mice were lightly anesthetized with isoflurane before 7.5 μl of the vaccine formulation was instilled into each nostril. NALT/nasal mucosa cells were isolated from five mice per group on days 34, 35, and 36, pooled, and analyzed via flow cytometry for the indicated cell-surface molecules.

<sup>b</sup> Statistically significantly greater than peptide only, p ≤ 0.05.

<sup>c</sup> Statistically significantly less than peptide only, p > 0.05. NT, not tested.
to be associated with the formulation of the Ag-cytokine mixtures and the route of immunization.

Nasal immunization with HIV immunogen and IL-1α, IL-12, and GM-CSF was associated with increased expression of MHC class II and B7.1 and B7.2 on nonlymphocytes within the NALT, although only the increase for MHC class II and B7.1 expression was statistically significant. Others have also reported that up-regulation of costimulatory molecules was observed when CT or mutant CT was used as a mucosal adjuvant (3, 45). When used to stimulate C57BL/6 T-depleted Peyer’s patch (PP) cells in vitro, mutant CT enhanced the expression of B7.2 and B7.1 on B220+ and Mac-1+ cells (45). Stimulation of C57BL/6 M-CSF-generated or GM-CSF-activated bone marrow macrophages with CT was associated with an increased expression of B7.2, but not B7.1 (3).

Gastric inoculation of C57BL/6 mice with 10 µg CT was associated with up-regulation of B7.2 expression on both Mac-1+ and CD11c+ cells isolated from the PP 24 h after the single CT treatment; the increase was statistically significant for the Mac-1+ cells only (3). It is not obvious why we observed significant up-regulation of B7.1 while others report a predominant up-regulation of B7.2. A difference between our experiment and the previously published work is that we used BALB/c mice, while the others used C57BL/6 mice. We also isolated cells from the NALT/nasal mucosal after nasal immunization, while the others utilized bone marrow macrophages or PP cells and gastric inoculation for their studies. We analyzed expression of costimulatory molecules with NALT/nasal mucosal cells isolated after four nasal immunizations with HIV immunogen and cytokine adjuvants, while others evaluated costimulatory molecule expression 24 h after intragastric inoculation or in vitro stimulation with CT or mutant CT, respectively (3, 45). It has been reported that B7.1 is more efficient than B7.2 for the induction of CD8+ T cell-mediated immune responses (46–48). Our vaccination regimen utilized an immunogen-adjuvant formulation that induced MHC class I-restricted, CD8+-mediated IFN-γ production and CTL. Therefore, it is possible that repeated immunization with the HIV immunogen and adjuvant formulation preferentially up-regulated expression of B7.1 for the induction of CD8+-mediated immunity. Thus, our study has demonstrated that cytokine combinations containing IL-1α, IL-12, IL-18, and/or GM-CSF could substitute completely for CT as a nasal adjuvant. It is important to note that up to 30 µg IL-1β administered i.n. to rabbits did not induce a febrile response (49). Thus, combinations of these cytokines are prime candidates for use as adjuvants with HIV and other vaccines.

Acknowledgments
We thank R. Dool for excellent technical assistance. We also acknowledge Dr. John F. Whitesides and Patrice M. McDermott of the Duke University Human Vaccine Institute and Department of Medicine Flow Cytometry Facility for excellent technical assistance with the flow cytometry.

References


